Analysis of T Lymphocyte Activation Measured by Super-Resolution Microscopy

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***Abstract*— Tight regulatory control of lymphocyte activation is necessary to avoid the deleterious consequences of an uncontrolled immune response. An intricate web of positive and negative regulation governs a key activation pathway in lymphocytes, the antigen-receptor-to-NF-κB pathway, particularly at a crucial signal transduction step mediated by a complex intracellular signaling structure called the POLKADOTS signalosome. However, the interplay between positive and negative control in this structure is currently poorly understood. We have utilized cutting-edge super-resolution imaging technologies combined with quantitative imaging analysis to examine the spatial organization of the POLKADOTS signalosome. Our preliminary results suggest that autophagosomes, small degradative organelles, localize preferentially to the ends of the filamentous POLKADOTS structure. Quantitative analyses of fixed cells images lend support to these initial imaging observations. Together, these results provide new insights into the complex regulatory processes which govern T lymphocyte activation.**

***Index Terms*— Super-Resolution Microscopy, T Lymphocyte, Signaling, Betweenness Centrality, Translocation I.**

I. Introduction

The antigen-receptor-to-NF-κB signaling pathway is a crucial mediator of T cell- and B cell-mediated adaptive immunity. Recognition of antigen by the cell surface antigen receptor triggers a series of signal transduction events that ultimately leads to the activation of the transcription factor NF-κB [1]. Activated NF-κB translocates to the nucleus, where it promotes transcriptional programs that control proliferation and effector differentiation of T cells and B cells [2]. While the central importance of the NF-κB pathway to the adaptive immune response is recognized, the exact mechanisms responsible for positive and negative regulation of this signaling pathway remain poorly understood. We have previously identified an intermediary signaling complex in antigen-receptor-to-NF-κB signaling pathway called the POLKADOTS signalosome [3,4]. This complex consists of long filaments comprised of the proteins Bcl10 and Malt1, which together promote further signaling events which eventually lead to translocation of the transcription factor NF-κB to the nucleus, where it can promote the expression of genes associated with lymphocyte activation and proliferation [2]. Intriguingly, the POLKADOTS signalosome filaments also recruit autophagosomes, intracellular vesicles associated with protein degradation [5,6]. Thus, POLKADOTS filaments promote both overall T lymphocyte activation and their own destruction, ensuring the kinetic control of the activation signal. While we have thus identified that positive and negative control mechanisms exist simultaneously in the same signalosome, we have a limited understanding of how these positive and negative control mechanisms are balanced to ensure highly precise activation of NF-κB by the antigen receptor.

In this paper, we begin to explore this question of balance between positive and negative control mechanisms by studying the co-localization of POLKADOTS filaments with autophagosomes, which negatively regulate POLKADOTS filament stability. Our super-resolution imaging of POLKADOTS filaments and autophagosomes in activated murine T lymphocytes appears to show a preferential attachment

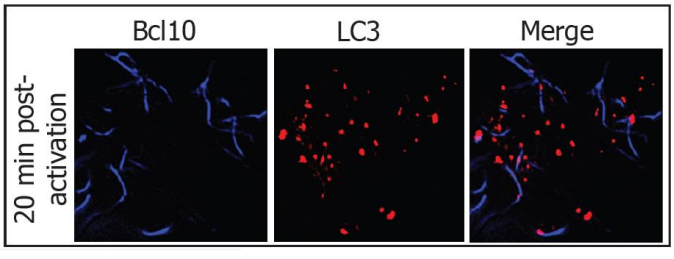


Figure 1. Autophagosomes colocalize with POLKADOTS filament ends. D10 cells expressing the proteins Bcl10-EGFP and LC3-TagRFP-t were incubated on coverslips coated with αCD3 antibody for 20m. Cells were then fixed and processed for imaging.

of autophagosomes to the filament ends. Extensive quantitative analyses of skeletonized images provide support for this conclusion. Together, these data suggest that negative control of T lymphocyte activation is achieved by preferential degradation of filament ends, and lends preliminary support to the hypothesis that positive and negative control mechanisms are spatially segregated in the POLKADOTS signalosome.

II. Super-Resolution Imaging of T Lymphocyte Activation

In previous experiments, we established via conventional confocal optical microscopy that the POLKADOTS signalosome contains, among others, the proteins Bcl10, Malt1, and LC3 (a structural component of autophagosome membranes), along with other protein factors which further transmit the activation signal [3-6,8]. This striking co-localization between positive and negative regulators of T lymphocyte activation lead us to postulate that POLKADOTS filaments have an underlying substructure which spatially separates positive and negative regulators. However, the resolution limitations of confocal microscopy did not allow us to ascertain further details about the structure of these signalosomes. We thus undertook imaging studies of the murine T helper 2 (TH2) cloned cell line, D10, utilizing a specially designed microscope capable of a modified version of the super-resolution imaging technique called Structured Illumination Microscopy (SIM) [7,9]. This imaging technique allowed us to obtain much higher resolution images of the POLKADOTS signalosome in fixed cells. These images appeared to show that, consistent with our hypothesis, autophagosomes localized nearly exclusively to the ends of POLKADOTS filaments, indicating that only a subregion of the POLKADOTS filament is devoted to negative regulation (Figure 1). We next decided to perform quantitative analysis of this set of images to further test the finding that autophagosomes preferentially bind to the POLKADOTS filament ends.

III. Quantitative Analysis of Super-Resolution Images

In order to study this co-localization of autophagosomes along the Bcl10 filaments, we implemented a multi-step analysis process to extract, quantify, and compile the data from the super-resolution images.

The extraction method consisted of elementary image analysis processes, such as intensity based threshholding and background subtraction using large Gaussian convolution kernels, and eventually culminated with 3D Alpha Shape rendering to best approximate the shape of the resulting extracted structures. Due to imaging irregularities it was found to be essential for there to be constant user oversight throughout the extraction process. To achieve this, a GUI allowing the user

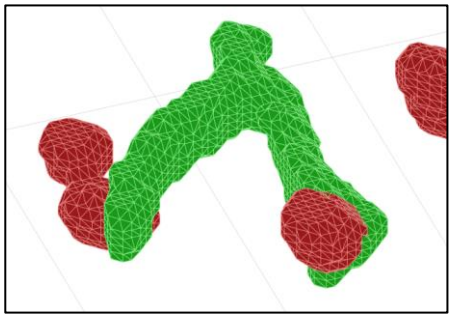


Figure 2. An extracted Bcl10 filament (green) with several small autophagosomes (red) localized to it.

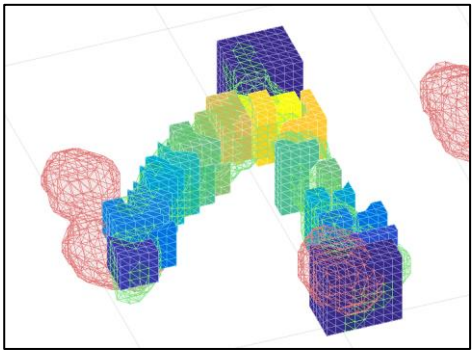


Figure 3. The same extracted Bcl10 filament shown in Fig. 2 except with the different betweenness centrality subregions colored by value: $\rho_k = 0 \rightarrow$ Dark Blue $\rho_k = 1 \rightarrow$ Yellow

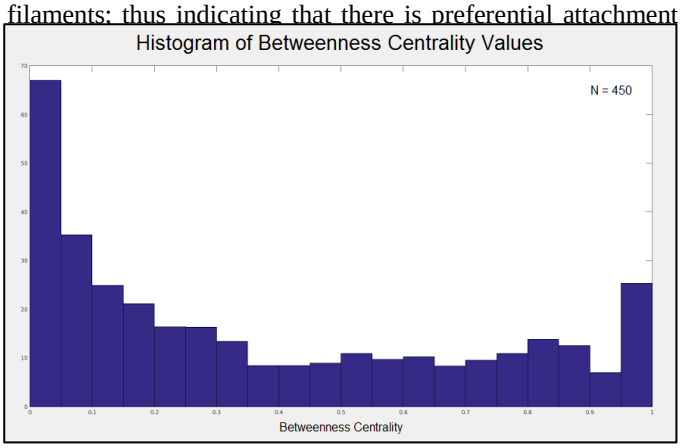
to fine tune the extraction parameters was created. An example output from the extraction technique is displayed in Fig. 2.

Following extraction, Kollmansberger's implementation of Blum's medial axis thinning algorithm was used to skeletonize the resulting Bcl10 filaments and with the resulting skeletons we calculated the weighted adjacency matrices [10,11]. Given this data, a metric was needed to best quantify the locations of autophagosome localization along Bcl10 filaments. We found the metric with the optimal set of properties to be Linton's betweenness centrality. Defined as:

$$\rho_k = \sum_{i,j} \frac{\sigma_{ij}(k)}{\sigma_{ij}} \quad (1)$$

where, in an unweighted graph, σ_{ij} is the number of shortest paths between i and j, and $\sigma_{ij}(k)$ is the number of shortest paths between i and j containing node k, the betweenness centrality is a scale-free metric, meaning $\rho_k \in [0,1]$, and therefore allows us to easily compare localizations along many different filaments despite filament size varying greatly from structure to structure and cell to cell (in weighted graphs we substitute σ as length instead of count) [12]. The resulting output simply labels the skeleton nodes on a particular filament in the following convention: $\rho_k = 0 \rightarrow \rho_k$ is a skeleton endpoint, $\rho_k = 1 \rightarrow \rho_k$ is the central node of a skeleton, $\rho_k \in (0,1) \rightarrow \rho_k$ is in the body of a skeleton between the center and an endpoint. An example result of this technique can be seen in Fig. 3.

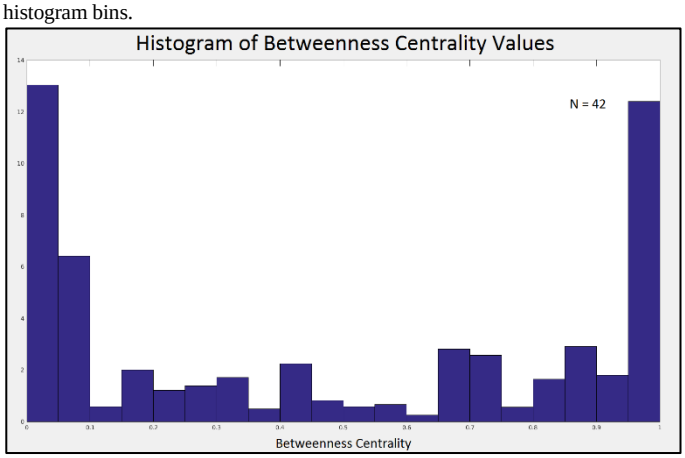
Once a localization is found the value(s) of the sections of filaments where overlap occurs are binned together to create a histogram (Fig. 4). As seen in the resulting histogram, there is significantly more overlap between the two structures in regions where $\rho_k < 0.1$ than there is anywhere else along the filaments; thus indicating that there is preferential attachment



of the autophagosomes to the ends of Bcl10 filaments.

Figure 4. The resulting localization histogram of autophagosome attachments to Bcl10 filaments. The x-axis is a set of histogram bins for the betweenness centrality values where localizations take place and the y-axis represents the count of those histogram bins.

Figure 5. Null Model Localization Analysis as described previously in Section III. The x-axis is a set of histogram bins for the betweenness centrality values where localizations take place and the y-axis represents the counts of those histogram bins.



IV. Discussion of Results Against the Null Model

Thus far we have demonstrated that there is a certain likelihood for there to be attachment of autophagosomes to particular subregions along Bcl10 filaments, however what is not shown is whether this result is biological or due to random interactions between the subcellular structures. We will dedicate the next section of this work to the comparison between the results obtained thus far and those obtain by generating a "Null Model" of Activated T Lymphocytes: a set of cells where the locations of the autophagosomes are solely governed by random chance.

To create the null model we generated autophagosomes of random sizes and shapes based on the experimentally observed distributions, and randomly placed them into the subcellular domain where they could be located based on the background fluorescence emitted in the images. After randomly placing these shapes in cells with the already existing set of Bcl10/POLKADOTS filaments, we repeated the localization analysis described in section IV of this work. The results of this procedure are shown in Fig. 5.

We find that the number of localizations within real cells is a factor of ten higher than in the null model. Compared to the 450 localizations measured in the real cell data, we observe a greater than tenfold decrease in the number of localizations in the null model; those cells only managed to achieve 42 random localizations. Furthermore, when grouping the results into three bins: $\rho_k < 0.05$, $0.05 < \rho_k < 0.95$, $0.95 < \rho_k$ the resulting histogram is nearly uniform across the entire domain, indicating that it is equally as likely for the autophagosomes in the null model to be attached to the endpoints as they are to the center of filaments.

V. Conclusion

We have developed a novel quantitative analysis tool for defining interactions between spherical and filamentous structures and utilized this tool to analyze super-resolution images of activated T lymphocytes. The resulting data suggests that autophagosomes preferentially localize to the ends of Bcl10/POLKADOTS filaments. At this stage, the analysis supports our hypothesis that the POLKADOTS signalosome is divided into positive and negative regulatory subdomains, however, further investigation of cells under additional experimental conditions will be required to rule out alternative models. We introduce betweenness centrality as a valuable measure for biological colocalization studies of filamentous structures. Given the importance of cytoskeletal filamentous structures to a wide variety of biological phenomena, we feel this methodology could have a wide area of potential use. Future studies will utilize a multi-channel SIM technique to address both the colocalization of positive regulatory factors with POLKADOTS filaments and the hypothesized spatial separation of positive and negative regulators.

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